Measurement of Serum Folate Levels and Serum Folic Acid-binding Protein by \(^{\text{3}}\)H-PGA Radioassay

By Samuel Waxman and Carol Schreiber

A radioassay for the measurement of serum folate levels using commercially available beta lactoglobulin, as the folate-binding protein, and \(^{3}\)H-pteroylglutamic acid (\(^{3}\)H-PGA) is reported. The assay was run in a one-step simultaneous addition at room temperature. A standard curve was constructed to a sensitivity of 0.25-10 ng of N-methyltetrahydrofolic acid (methyl-THFA). There is a clear correlation with separation into normal (greater than 6 ng/ml), indeterminate range (3-6 ng/ml), and deficient (0-3 ng/ml), as measured by radioassay. Serums from patients receiving antibiotics had normal folate levels with this assay. \(^{3}\)H-PGA was also used to measure serum folic acid-binding protein (FABP). In normal serums, the mean FABP was 18 pg bound/0.4 ml serum, while in folate-deficient serums it was 133 pg bound/0.4 ml of serum. Folate-deficient patients had a fall in serum FABP to the normal range when treated with folate acid. Serum FABP, in a patient on a folate-deficient diet, increased with early folate deficiency and abruptly fell to normal with a regular diet. FABP in lysates of folate-deficient bone marrow was higher than normal marrow. Patients with \(\text{B}_{12}\) deficiency, multiple myeloma, cirrhosis, pregnancy, or taking oral contraceptives had normal FABP. Elevated FABP was found in two out of ten patients taking Dilantin. This radioassay and the measurement of FABP should simplify the diagnosis of folate deficiency.

RECENTLY, we reported a radioassay for the measurement of serum folate levels using specially prepared, high specific activity \(^{3}\)H-N-5-methyltetrahydrofolic acid (\(^{3}\)H-methyl-THFA) and commercially available powdered cow's milk.\(^1\) We also reported a modification of that assay whereby serum folate levels were measured using commercially available, high specific activity \(^{3}\)H-pteroylglutamic acid (\(^{3}\)H-PGA) in a two-step sequential method.\(^1,2\) We now report the use of beta lactoglobulin (BLG), the folate-binding protein of milk,\(^3\) with \(^{3}\)H-PGA for the measurement of serum folate levels. BLG, as a binder for this assay, is a commercially available crystalline material that is stable, easy to work with, and allows the assay to be run in a rapid one-step simultaneous addition at room temperature.

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Folate Materials

$^3$H-PGA, purchased from Amersham-Searle, Des Plaines, Ill. (specific activity, 40 Ci/mM), was stored in concentrated form at $-70^\circ$C in amber tubes. Working concentrations (5 ng/ml) of $^3$H-PGA were prepared in phosphate buffer in convenient aliquots and were thawed before use. In our experience, $^3$H-PGA is usually greater than 90%, pure, but because of the endogenous radioactivity, it deteriorates slowly (8-12 wk) even when carefully stored. Therefore, we advise replacing the material periodically with a freshly prepared new batch. Purity of $^3$H-PGA should be assayed by descending paper chromatography using stable PGA as a marker in 0.1 M phosphate buffer, pH 7.4. Stable d, L-methyl-THFA was obtained from Sigma Chemicals, St. Louis, Mo. and was stored with 2-mercaptoethanol (ME) at $-70^\circ$C in covered tubes. Methyl-THFA should be purified by DEAE-cellulose chromatography and should be assayed for purity by analysis of its absorption spectra and by microbiologic assay with Streptococcus faecalis compared to Lactobacillus casei. The methyl-THFA was greater than 99%, pure after the DEAE-cellulose chromatography. Deterioration, probably oxidation, of methyl-THFA became evident by a marked increase in sensitivity of the standard curve. All reduced forms of folic acid were stored in saline containing 2-mercaptoethanol at $-70^\circ$C.

Beta Lactoglobulin ($^3$H-PGA Binder)

Crystalline BLG was obtained from Sigma Chemicals, St. Louis, Mo. BLG working solutions (1 mg/ml) were prepared in 0.1 M phosphate buffer, pH 7.4, and were stored frozen ($-10^\circ$C) in aliquots. This material is stable and did not lose $^3$H-PGA-binding activity after 3 mo or after freeze-thawing.

Hemoglobin-coated Charcoal

Hemoglobin-coated charcoal (1:20 ratio hemoglobin to charcoal) was prepared and used as previously described. Serum and bone marrow were obtained from fasting normals and from patients with folic acid deficiency, pregnancy, cirrhosis, jaundice, multiple myeloma, and those taking oral contraceptives and were assayed for binding of $^3$H-PGA. In some studies, serial blood samples were obtained from the same patient prior to, during, and after treatment for folic acid deficiency. Bone marrow was prepared by dextran sedimentation followed by hypotonic shock to remove red cells. The cell buttons were suspended in phosphate buffer and were freeze-thawed three times. Lysates were centrifuged at 5000 rpm, and the supernates were assayed. Freeze-thawing did not diminish the $^3$H-PGA-binding capacity.

Incubation Mixture

The incubation mixture for assay of serum folate levels (Table 1) consisted of the simultaneous addition of 0.4 ml serum, 0.1 ml $^3$H-PGA (0.5 ng), 0.1 ml saline-ME, 0.6 ml 0.1 M Na-KPO$_4$ buffer (pH 7.4), and 0.1 ml BLG (concentration determined from BLG binding-capacity curves) (Fig. 1). The concentration of BLG used for assay should bind approximately 60%, 70%, of 0.5 ng of $^3$H-PGA. In our experiment, this was 0.1 mg BLG diluted with phosphate buffer. The mixture was equilibrated at room temperature for 30 min.

Separation

At the end of the incubation period, the free (unbound) $^3$H-PGA was separated from that which was bound by 15-min contact with 1.0 ml of the hemoglobin-coated charcoal suspension. Following centrifugation, an aliquot of the clear supernate containing the bound $^3$H-PGA was added to
Table 1. Serum Folate Assay Protocol (Values in Milliliters)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Saline ME*</th>
<th>Unknown Serum</th>
<th>Methyl-THFA (0-100 ng/ml)</th>
<th>0.1 M PO4 Buffer, pH 7.4</th>
<th>3 H-PGA (5 ng/ml)</th>
<th>Blg</th>
<th>Hb-Coated Charcoal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corrected standard (STD)</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>2.1</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Buffer supernatant control (SC)</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
<td>1.1</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Standard curve</td>
<td>0</td>
<td>0</td>
<td>0.1*</td>
<td>1.0</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Unknown serum</td>
<td>0.1</td>
<td>0.4</td>
<td>0</td>
<td>0.6</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Serum supernatant control (SSC)</td>
<td>0.1</td>
<td>0.4</td>
<td>0</td>
<td>0.6</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>Serum-binding control (SBC)</td>
<td>0.1</td>
<td>0.4</td>
<td>0</td>
<td>0.7</td>
<td>0.1</td>
<td>0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*0.85% NaCl containing 3.7 X 10^{-7} M 2-mercaptoethanol
†Mix: 30 minutes at room temperature.
‡Mix: centrifuge after 15 minutes and count supernatant
§0.1 ml methyl THFA in saline ME in increasing amounts from 0-10 ng is added to construct standard curve.

0.1 ml scintillation mixture (toluene containing 0.7% 2,5-diphenyloxazole (PPO) and 10% bio-SOLV BBS-3 (Beckman Instruments, Fullerton, Calif.) and was counted in a Beckman Model 250 Scintillation Counter.

**Serum Supernatant Control (SSC)**

A serum supernatant control was run with each serum sample to determine any radioactivity that was not adsorbable to charcoal in the absence of BLG. Stable PGA (5 ng) was added to the mixture to block serum binding of 3H-PGA. The remaining 3H-PGA activity not adsorbable to charcoal represented impurities of 3H-PGA and was subtracted from the 3H counts of each serum incubation mixture.

**Standard (STD)**

The corrected standard (STD) was the amount of radioactivity present when the test amount (0.5 ng) of 3H-PGA was measured in the absence of BLG and of charcoal, minus the buffer supernatant control (SC). When standard curves were constructed (Fig. 2), serum was replaced by stable methyl-THFA (0-10 ng) and phosphate buffer in appropriate quantities, and was assayed as described above (Table 1). The standard curves obtained were linear when plotted 1/[^bound] bound.

**Calculation of Serum Folate Level**

The 1/[^bound] bound ratio was calculated for each serum assayed, and the serum concentration of methyl-THFA was determined by reference to the standard curve.
Assay for Measurement of Folic Acid-binding Protein (FABP)

The FABP was directly measured from the serum supernatant control (SSC) tube by adding a single tube for each serum assayed designated the serum-binding control (SBC), where phosphate buffer was added instead of stable PGA. Thus, FABP represents the amount of $^3$H-PGA bound to serum (SBC) minus the radioactivity remaining in the SSC divided by the radioactivity in the STD.

Fig. 2. Standard curve prepared by simultaneous addition of d,l-methyl-THFA (0-10 ng) and 0.5 ng $^3$H-PGA to BLG (0.1 mg). The 1/% bound ratio of $^3$H-PGA is plotted against added d,l-methyl-THFA.

Fig. 3. Comparison of folate levels of 80 serums assayed by L. casei and $^3$H-PGA-BLG radioassay. Deficient, indeterminate, and normal range levels refer to the radioassay.
MEASUREMENT OF SERUM FOLATE LEVELS

MEASUREMENT OF SERUM FOLATE LEVELS

RESULTS

Figure 3 shows a comparison of 80 serum folate levels measured by both the aseptic addition L. casei method and the 3H-PGA-BLG assay, as separated into deficient, indeterminate, and normal areas based on the results of the radioassay. Each serum was tested in duplicate. There was a close correlation in the serums studied and a definite separation without overlap between low (0-3 ng/ml) and normal (greater than 6 ng/ml) serum folate levels. An indeterminate range (3-6 ng/ml) was present. The L. casei folate levels tended to be 15-20% higher than those obtained by radioassay. The folate levels obtained from each serum by the two assays were sufficiently similar to permit the same diagnostic interpretation.

Deterioration of the serum folate activity measured by the radioassay was significant (80% decrease) in 3 days, if the serum was allowed to stand at room temperature, but not significant after 4 wk at -10°C. This deterioration was prevented by the addition of ascorbic acid to serum (5 mg/ml). Serum dialyzed overnight against 0.1 M phosphate buffer, pH 7.4, lost 90% of folate activity when measured with the radioassay. Deproteinated extracts of serum prepared by boiling for 15 min in ascorbate and phosphate buffer had essentially the same folate level as serums not extracted (Table 2). Recovery of added methyl-THFA to dialyzed (folate-free) serum high in FABP was as expected (Table 2).

Serums obtained from patients receiving antibiotics were measured for folate levels by both assay methods. In seven such serums, where L. casei levels were in the deficient range (presumably antibiotic interference with L. casei growth), normal values were obtained with the radioassay.

The effect of various folate analogues on the binding of 3H-PGA by BLG is shown in Fig. 4. Oxidized folates (including the folyl polyglutamate pteropin) compete for BLG binding better than the reduced folate analogues. Methyl-THFA competes more than methotrexate or formyl-THFA. Vitamin B12 (in ng amounts) does not inhibit BLG binding of 3H-PGA.

The relationship between serum FABP and serum folate levels is shown in Fig. 5. The serums were divided into folate-deficient (less than 5 ng/ml) and definitely normal (more than 10 ng/ml). Each serum was run in duplicate for both folate level and FABP. The mean FABP in 25 folate-deficient serums was 26.7% of 0.5 ng 3H-PGA added, or 133 pg bound/0.4 ml serum. In 25 normal serums, the mean FABP was 3.6% of 0.5 ng 3H-PGA added, or 18 pg bound/0.4 ml of serum. The difference in mean FABP between the normal and folate-deficient groups was significant to a p value of less than 0.001.

Five folate-deficient patients were studied prior to and following treatment with folic acid. The serum FABP fell from the folate-deficient range to the normal range immediately following folic acid replacement (Fig. 5). The serum FABP in the postfolic acid-treated patient remained in the normal range despite 24-hr dialysis against saline, which reduced the serum folate level from 25 ng/ml to 1.5 ng/ml. Therefore, the fall in serum FABP following folic acid replacement did not result from dilution of 3H-PGA but was due to saturation of the serum FABP by the stable folic acid given to the patient.
Table 2. Effect of Serum FABP on the Measurement of Serum Folate Levels

<table>
<thead>
<tr>
<th>Added to Serum (ng)</th>
<th>Folate Assayed (ng)</th>
<th>% Expected Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.97</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>1.6</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>3.6</td>
<td>90</td>
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<tr>
<td>6</td>
<td>5.4</td>
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</tr>
<tr>
<td>8</td>
<td>6.1</td>
<td>77</td>
</tr>
<tr>
<td>10</td>
<td>11.2</td>
<td>112</td>
</tr>
</tbody>
</table>

Comparison of Folate Concentrations Obtained by Radioassay of Whole Serum and Serum Extracts

<table>
<thead>
<tr>
<th>Serum FABP Content</th>
<th>L. Case (ng/ml)</th>
<th>Whole Serum (ng/ml)</th>
<th>Serum Extract (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>11.5</td>
<td>10.5</td>
<td>9.6</td>
</tr>
<tr>
<td>5.7</td>
<td>9.5</td>
<td>8.6</td>
<td>11.5</td>
</tr>
<tr>
<td>45.1</td>
<td>2.5</td>
<td>2.7</td>
<td>3.3</td>
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<tr>
<td>48.6</td>
<td>1.3</td>
<td>1.3</td>
<td>4.0</td>
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<td>19.4</td>
<td>3.2</td>
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<td>4.1</td>
</tr>
<tr>
<td>13.6</td>
<td>5.5</td>
<td>4.3</td>
<td>5.0</td>
</tr>
<tr>
<td>52.0</td>
<td>1.35</td>
<td>1.62</td>
<td>1.95</td>
</tr>
</tbody>
</table>

*FABP content = 33%*

FABP was studied in frozen serum specimens obtained from a patient on a folate-deficient diet. The patient, a 28-yr-old 400 lb black man with Pickwickian syndrome and alveolar hypoventilation, was placed on a low calorie diet on June 8, 1966, resulting in a weight loss of 90 lb. When weight reduction slowed on October 15, 1966, a folate-deficient diet was combined with the low calorie diet. Low serum folate levels and a rise in serum FABP were evident within 6 wk and remained so, except on March 13, 1967 when a regular meal was given, which resulted in a rise in serum folate and an abrupt fall in...
Fig. 5. Relationship of serum FABP to serum folic acid levels. The lines connect the FABP and serum folate level of the same patient prior to and following folic acid replacement.

Fig. 6. Serum FABP and folate levels of a patient placed on a folate-deficient diet followed by a regular diet.
Fig. 7. $^3$H-PGA-binding capacity of 0.4 ml of various serum: 0.5 ng of $^3$H-PGA added offers the greatest difference in levels between FABP of folate-deficient and normal serum.

serum FABP. On May 30, 1967, after a further weight loss of 75 lb, the patient was placed on a 1000 calorie normal diet with folic acid replacement. A rise in serum folate to the normal range and a fall in serum FABP promptly occurred.

The $^3$H-PGA binding capacity of 0.4 ml of various sera are represented in Fig. 7. The binding appears biphasic, and if more than 0.5 ng of $^3$H-PGA is added there is a plateau in the amount of $^3$H-PGA bound. The increase in $^3$H-PGA-binding capacity (FABP) of folate-deficient serum above normal FABP was most apparent at 0.5 ng of added $^3$H-PGA and diminished with increasing amounts of added PGA. Thus, an optimum serum and PGA ratio is essential and may explain why the difference in folate-deficient and normal FABP was not evident when large amounts of $^3$H-PGA were added to the serum, or when too little serum was used.

Bone marrow lysates obtained from normal and folate-deficient patients were measured for FABP. Bone marrow FABP, as found with serum FABP, was much greater in the folate-deficient patients than in normal subjects (Table 3).

Several patients with multiple myeloma (3), cirrhosis (7), jaundice (7), pregnancy (9), or those taking oral contraceptives (5), Dilantin, or phenobarbital, were studied. All of these patients had normal serum folate levels and serum FABP, except for two patients taking Dilantin who had FABP of 47.2% and 20.9%, with low normal serum folate levels.

Table 3. Levels of FABP in Normal and Folate-deficient Bone Marrow

<table>
<thead>
<tr>
<th></th>
<th>Serum Folate* (ng/ml)</th>
<th>Serum FABP† (pg $^3$H-PGA Bound/0.4 ml Serum)</th>
<th>Bone Marrow Lysate FABP† (pg $^3$H-PGA Bound/ mg Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. S.</td>
<td>15.5</td>
<td>26.5</td>
<td>56.0</td>
</tr>
<tr>
<td>H. P.</td>
<td>13.5</td>
<td>7.5</td>
<td>7.0</td>
</tr>
<tr>
<td>S. D.</td>
<td>11.0</td>
<td>18.0</td>
<td>74.0</td>
</tr>
<tr>
<td>Folate deficient</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R. M.</td>
<td>2.5</td>
<td>133.0</td>
<td>155.0</td>
</tr>
<tr>
<td>R. D.</td>
<td>1.8</td>
<td>87.0</td>
<td>238.0</td>
</tr>
</tbody>
</table>

* L. casei method.
† $^3$H-PGA added, 500 pg.
DISCUSSION

Serum folate levels have been shown to be an accurate index of body stores of folate and rapidly fall in the deficient state. Thus, microbiologic assay of serum folate is a routine procedure in most hospitals throughout the world. However, this assay cannot be used easily with turbid or bacterially contaminated serum and gives false low values if the serum contains high levels of certain antibiotics or antifolates. Microbiologic assay is tedious and requires the maintenance of a special laboratory for this purpose. We recently reported a radioassay for measurement of serum folate levels without these disadvantages but with the requirement of an isotope not as yet commercially available. At that time, we also reported studies of a modification utilizing commercially available 3H-PGA in a more complicated two-step sequential assay.

Present studies using commercial crystalline BLG have resulted in a 3H-PGA assay that is rapid (4 hr), reproducible (within 10%), and sensitive (0.25–10 ng) assay for serum folate levels. It requires a simultaneous addition, since methyl-THFA bound to BLG is not displaced by 3H-PGA during the time of assay and can be measured by the inhibition of 3H-PGA binding to BLG. BLG can be stored frozen, and reproducible linear standard curves are easily prepared. The purity of 3H-PGA and methyl-THFA must be carefully followed, since these materials are subject to deterioration with time. The presence of folic acid-binding protein does not appear to affect the determination of the serum folate level, as demonstrated by serum extraction and methyl-THFA recovery experiments. This is probably so because the folate assay is run using an amount of BLG near the range of maximal binding of the added 3H-PGA.

The 3H-PGA-BLG assay and the microbiologic assay separate serum folate levels into similar diagnostic groups. Red cell or whole blood folate levels can also be determined by the 3H-PGA—BLG assay by preparation of hemolysates by freeze-thawing.

A radioassay for serum folate similar to our original assay has recently been reported. That assay utilized a more complicated sequential assay with partially purified milk binder and the assay was terminated at 4°C. BLG obviates the need for milk purification and allows the use of simultaneous addition and the construction of a linear standard curve. Moreover, the assay is not significantly influenced by 4°C incubation.

3H-PGA can be used to measure serum folate-binding protein (FABP) by a simple addition of another set of test tubes to the 3H-PGA—BLG assay. FABP is a specific binding protein for folate that we have characterized and that is reported in a separate paper. FABP must be measured with a standard amount of 3H-PGA per test amount of serum (0.5 ng/0.4 ml serum), since its binding specificity can be obscured by excess PGA. This may explain why previous attempts using larger amounts of 3H-PGA failed to recognize FABP.

FABP is measurable in eight times greater quantity in folate-deficient serum as compared to normal serum. The FABP level rises early in folate deficiency and returns to normal with folate replacement. FABP levels are also greater in folate-deficient than in normal marrow. Preliminary studies have not revealed levels of serum FABP comparable to that of folate deficiency in normal (non-folate-deficient) subjects or in patients with multiple myeloma, cirrhosis,
jaundice, pregnancy, or in those taking oral contraceptives. However, it will be necessary to study a large population in order to establish serum FABP levels as an important parameter of clinical folate stores.

ACKNOWLEDGMENT

We wish to thank Miss Leona Bandel and Mrs. Z. Bokslawski for measurement of serum folates by *L. Casei*, and Dr. Leslie Bernstein for assaying folates with *Streptococcus faecalis*. We are indebted to Mr. Peter Kosovsky for his technical assistance. The frozen serum specimens obtained from the patient on a folate-deficient diet were kindly provided by Dr. Victor Herbert.

REFERENCES

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